REMARKS

This application has been amended in a manner that is believed to place it in condition for allowance at the time of the next Official Action.

Claims 39-48 are pending in the present application. Claims 13, 14, 17, 21, 24, 33-35, 37, and 38 have been canceled. Support for new claims 39-48 may be found generally throughout the specification and in original claims 13, 14, 17, 21, 24, 33-35, 37 and 38.

In the outstanding Official Action, the disclosure was objected to for containing an embedded hyperlink and/or other form of browser-executable code. As requested by the Examiner, the embedded hyperlink has been deleted from the specification. Thus, it is believed that the objection has been obviated by the present amendment.

Claims 13, 17, 21, 24, 33, 35, 37 and 38 were rejected under 35 USC §112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicants believe that the present amendment obviates these rejections.

The outstanding Official Action alleged that claims 13, 17, 21, 24, 33, 35, 37 and 38 were vague and indefinite for

reciting the term "capable of". In the interest of advancing prosecution, this term has been deleted from the new claims. Indeed, it is believed that the claims have been drafted in a manner that provides a positive recitation.

Claims 17 and 37 were believed to be vague and indefinite for reciting the term "molecular weight". The outstanding Official Action stated it was unclear how the molecular weight was determined. However, claims 41, 47 and 53 have drafted to recite the weight of the polypeptide in terms of kDa.

On page 37 of the specification, second paragraph, it is stated that the molecular weight has 2228 amino acids, from which the molecular weight can be estimated to 260 kDa. In addition, the polypeptide is identified by gel electrophoresis (see Figure 1 and page 28 in the present specification).

Claim 34 was objected to for containing several informalities. Claim 34 has been amended to depend from a It is believed that claims 39-48 have been drafted in a manner so as to eliminate these informalities.

Thus, it is believed that claims 39 and 48 are definite to one of ordinary skill in the art.

In the outstanding Official Action claim 14 was rejected under 35 USC §101. The Official Action alleged that the claimed invention was directed to non-statutory subject matter.

It is believed that the present amendment obviates this rejection.

As suggested by the Examiner, the polypeptide is recited in terms of an isolated polypeptide.

Indeed, applicants would like to thank the Examiner for her suggestions as to how to overcome this rejection and others.

Claims 24, 33, 34 and 38 were rejected under 35 USC §112, first paragraph, as allegedly being based on a non-enabling disclosure. This rejection is respectfully traversed.

In imposing the rejection, the Official Action alleged that the present specification is not enabling for a pharmaceutical, vaccine, or medicament comprising the polypeptide or any amino-terminal part of the polypeptide of SEQ ID No. 1.

Applicants note that claims 39-42, 46-51 are not directed to applicants do not disclaim. Claims 39-42, 46-47, and 48-51 are directed to an isolated polypeptide. Claim 48 is directed to a composition. Thus, claims 39-42, 46-47 and 49-51 are directed to an isolated polypeptide or composition and are not limited to pharmaceutical, vaccine, or medicament. Indeed, claims 39-42, 46-47 and 49-51 may be utilized in variety of applications.

As to claims 43, 44, and 45, applicants respectfully submit that the present specification clearly enables one of ordinary skill in the art how to make and use the claimed

invention. Indeed, the Examiner's attention is directed to the Chen al. article. For the development of a vaccine, the DBL-1 domain of the isolated polypeptide PfEMP1 the parasite FCR3S1.2 was selected. It was shown that antibodies generated by a vaccine recognized native pfEMP1 at the infected erythrocyte surface, disrupt rosettes and prevent the sequestration of *P. falciparum*-infected erythrocytes *in vivo*. The data shows that pfEMP1-DBL-1 induces rosette-disruptive antibodies that protect against the sequestration of infected RBC and DBL-1 is a vaccine against severe malaria.

The n-terminal DBL-1 domain of pfEMP1 exists in all of PfEMP1 molecules. In fact, it is believed that this portion of the sequence is the most conserved domain of the pfEMP1 domain. Moreover, applicants note that two independent studies confirm that FCR3S1.2 fr1/pfEMP1 is commonly recognized by antibodies from malarial immune serum (see Carson et al. and Chen et al.). For the Examiner's convenience, these articles have been included with the present amendment.

Indeed, applicants have discovered that immune-antibodies generated by a vaccination with recombinant pfEMP1-DBL-1 constructs recognize native PfEMP1 on a live infected red blood cell surface, disrupt preformed *P.falciparum* rosettes and hinder the adhesion of infected erythrocytes in an animal model.

Thus, in view of the above, it is believed that the present specification is enabling for the claimed invention.

Claims 13, 14, 17, 21, 14, 33-35, 37 and 38 were rejected under 35 USC §112, first paragraph, for allegedly being based on a non-enabling disclosure. The outstanding Official action alleged that the specification was non-enabling for a polypeptide comprising any part of the SEQ ID No. 1. This rejection is respectfully traversed.

Applicants note that the claimed invention is not directed to any part of SEQ ID No. 1. Rather, the claimed invention is directed to an isolated polypeptide originating from a malaria erythrocyte membrane protein of SEQ ID NO. 1. SEQ ID No. 1 is the sequence of the complete malaria erythrocyte membrane protein, pfEMP1. The isolated polypeptide consists of an amino-terminal part of SEQ ID NO. 1. Moreover, the claims recite that the amino-terminal part of the sequence comprises domain DBL-1. Thus, it is believed that it is clear that the claimed invention is not directed to just any portion of SEQ ID NO. 1. As a result, applicants respectfully request that the rejection be withdrawn.

Claims 13, 14, 24, 33-35, 37, and 38 were rejected under 35 USC §102(b) as allegedly being anticipated by HELMBY et al. This rejection is respectfully traversed.

Applicants respectfully submit that the polypeptides disclosed by HELMBY et al. fail to anticipate or render obvious the claimed invention. Indeed, polypeptides as described by HELMBY et al. are distinct from DBL-1. HELMBY et al. disclose the isolation of small proteins, termed rosettins. Their surface antigens of P.*falciparum* infected red blood cells. However, these rosettins (now know as rifins) are not actually involved in resetting. This is clearly demonstrated by Feranadez et al. (J. Exp. Med., 1999). In fact, these polypeptides have a molecular weight of 22 or 28 kDa. This stands in distinct contrast to the DBL-1 peptide of the claimed invention which has a molecular weight of 48 kDA.

Thus, it is respectfully submitted that HELMBY et al. fail to anticipate or render obvious the claimed invention.

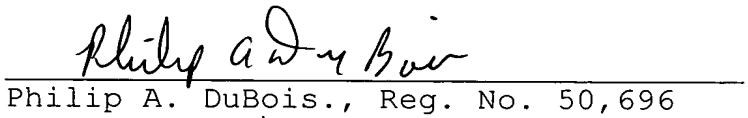
In view of the present amendment and the foregoing remarks, therefore, it is believed that the present application is now in condition for allowance, with claims 39-51, as presented. Allowance and passage to issue on that basis are accordingly respectfully requested.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any

overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

YOUNG & THOMPSON



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APPENDIX:

The Appendix includes the following publications:

- Carlson et al., "Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies", The Lancet, vol. 336, Dec. 15, 1990, pp. 1457-1460.

- Chen et al., "Severe malaria: immunization with PfEMP1-DBL1α protects against the sequestration of *Plasmodium falciparum*-infected erythrocytes, Manuscript, Medical Sciences, Proc. Natl. Acad. Sci.

MEDICAL SCIENCE

Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies

JOHAN CARLSON HELENA HELMBY ADRIAN V. S. HILL
DAVID BREWSTER BRIAN M. GREENWOOD MATS WAHLGREN

Plasmodium falciparum **isolates from 24 Gambian children with cerebral malaria and 57 children with mild forms of the disease were assessed for their ability to form erythrocyte rosettes. All isolates from the children with cerebral malaria were able to form rosettes, whereas those from children with mild forms of the disease did not form rosettes, or had a significantly lower rosetting rate. Plasma of children with cerebral malaria lack d anti-rosetting activity, whereas plasma of children with mild disease could often disrupt rosettes in vitro. A monoclonal antibody to *P falciparum* histidine rich protein (PfHRP1/KP/KAHRP) disrupted rosettes of many of the isolates in vitro indicating that the rosetting ligand is relatively conserved compared with ligands associated with endothelial cytoadherence. The findings strongly support the hypothesis that erythrocyte rosetting contributes to the pathogenesis of cerebral malaria and suggest that anti-rosetting antibodies protect against cerebral disease.**

Lancet 1990; **336**: 1457–60.

Introduction

Occlusion of cerebral vessels by tightly packed red blood cells is a characteristic post-mortem finding in the microvasculature of patients with cerebral malaria. Sequestration of infected red blood cells followed by occlusion of cerebral vessels has been suggested as the underlying mechanism. The binding of infected erythrocytes to the endothelial lining contributes to sequestration and may also take part in the development of cerebral disease. Recently, it has been suggested that rosetting (ie, adherence of uninfected red blood cells to *Plasmodium falciparum*-infected red blood cells) also has a role in erythrocyte sequestration and the formation of cerebral malaria. Erythrocyte rosetting was first described in *P falciparum* malaria;[1-3] frequently more than ten red blood cells could be found attached to a single infected cell and giant rosettes—ie, aggregates of as many as 20 infected and 40–50 uninfected red blood cells—were sometimes seen.[2,4] Rosetting parasites have also been found in other malarias (*P chabaudii*,[1] *P fragile*[5]) in which parasite sequestration is a characteristic of infection.

Rosette formation is a pH and heparin sensitive cell-to-cell interaction dependent on the presence of divalent cations (Ca^{2+}/Mg^{2+}).[6] Rosettes are also disrupted by both monoclonal and polyclonal antibodies to a *P falciparum* histidine rich protein (PfHRP1/KP/KAHRP; mab 89/rabbit serum MC1).[6] Anti-PfHRP1 antibodies primarily react with an antigen of 28 kD (immunoprecipitation) and weakly with an antigen of 90 kD (immunoblotting), but the target of the inhibitory antibodies is not known.[6] Anti-rosetting activity is also induced by natural infection: 20% of sera from individuals living in a malaria endemic area (Yekepa, Liberia) disrupt rosettes in vitro, whereas no anti-rosetting activity has been found in the sera of individuals from a non-endemic area (Sweden) infected with other parasites or viral pathogens.[7]

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We have proposed that rosetting is involved in the pathogenesis of severe malaria.[1,4,6,8] We have now studied the rosetting phenotypes of *P falciparum* isolates from children with both severe and mild disease and corresponding sera for their ability to disrupt rosettes in vitro.

Patients and methods

Patients

Between Oct 5 and Nov 17, 1989, all patients with a positive malaria smear (*P falciparum*) and signs and symptoms suggestive of acute malaria were admitted to the Royal Victoria Hospital, Banjul or the Medical Research Council Laboratories, Fajara, The Gambia. There were two groups of patients included in the study: (1) those with cerebral malaria—ie, unrousable coma (modified Glasgow coma scale score 2 or less[9]) for more than 6 h where other causes of cerebral disease had been excluded; and (2) uncomplicated malaria (mild disease)—ie, absence of cerebral malaria, severe anaemia (haemoglobin <5 g/dl), hypoglycaemia (glucose $<2{\cdot}2$ mmol/l), or other indices of severe disease.

Culture of P falciparum *and assessment of erythrocyte rosetting*

Blood was taken from the patients; parasites were cultured according to standard procedures[10] at 5% packed cell volume, with 10% heat-inactivated human AB^+Rh^+ serum added to the buffered medium (pH about 7·2). Plasma samples from each patient were stored at -20°C until used. Strains that grew until schizogony were included in the study and assessed for the ability to form erythrocyte rosettes.[6] 30 of the patients with cerebral malaria did not have a history of chloroquine consumption and 67% (20/30) of the parasites from those patients grew in vitro. Of the 79 uncomplicated cases without a stated history of chloroquine intake 70% (55/79) yielded parasites. Only a small proportion of isolates from patients with a stated history of chloroquine intake before admission grew adequately (7% [4/54] in the cerebral malaria group and 11% [2/18] in the group with uncomplicated disease). Since the mean rosetting rate of these isolates differed very little (<1%) from the mean rosetting rate of each group, inclusion of these rosetting rates with the other results did not affect the observed differences between the two groups. Thus, a total of 81 samples (24 from patients with cerebral malaria and 57 from patients with mild or moderate, uncomplicated disease) were available for study.

Assessment of rosetting was made after 18–24 h, 30–36 h, and 48 h (and later if possible). A sample of the parasite culture was mixed with a small amount of 0·001% acridine orange, mounted on a glass slide, and 50 consecutive fields were counted with a 40 × lens in incident ultraviolet light as previously described.[6] Infected erythrocytes that had bound two or more non-infected ones were scored as rosettes and the rosetting rate was expressed as the number of infected erythrocytes in rosettes relative to the total number of late stage (trophozoite and schizont) infected erythrocytes. Thus, a maximum rosetting rate for each isolate, usually obtained in the late trophozoite phase (about 30 h) of the asexual life cycle, was calculated.

Disruption of rosettes of fresh clinical isolates

Studies were done on fresh clinical isolates kept in standard culture for 30–36 h. 25 µl of the rosetting *P falciparum* culture was mixed with equal amounts of (a) mab 89 (IgG_{2a}) (mouse monoclonal antibodies to PfHRP1/KP/KAHRP, histidine rich protein 1/knob protein/knob associated histidine rich protein)[6,11] or (b) human immune serum BD 245 (showing strong anti-rosetting activity on screening and obtained from a healthy blood donor living in Yekepa, a malaria endemic area of Liberia)[7] at final dilutions of 1/10 and 1/2. After incubation at 37°C for 60 min assessment for rosetting was made. The rosetting rate of each well was compared with that of a control (serum from a healthy Swedish blood donor).

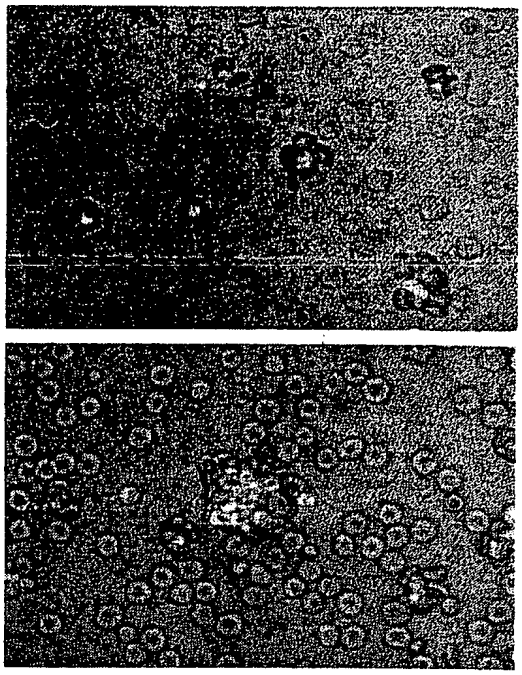

Fig 1—Erythrocyte rosetting.

Spontaneous binding of uninfected erythrocyte to *P falciparum*-infected erythrocytes appears in the late trophozoite stage and disappears when the schizonts rupture. Upper, rosetting with one, sometimes two or three, infected red blood cells surrounded by uninfected ones. Lower, "giant" rosette consisting of a large number of infected and uninfected erythrocytes.

Disruption of rosettes with plasma from the diseased children

The ability of individual plasma samples to disrupt rosettes was tested at a final dilution of 1/10 with a cloned highly rosetting in-vitro-propagated *P falciparum* isolate (R+PA1).[2] Rosette disruption of at least 15% (mean of controls ±2 SD) was regarded as significant.[7] Since rosetting has been shown to be affected by heparin,[2,6] heparin was removed from the plasma samples with an ion-exchange matrix (Heparsorb', Organon Teknika Corporation, North Carolina, USA). Heparsorb, when used in our system, was found to effectively deplete heparin from sera and did not negatively affect sera previously known to contain anti-rosetting antibodies (data not shown).

Results

Infected blood was obtained from 24 Gambian children with unrousable coma (mean age 4·5 years, range 0·5–12) and 57 children with uncomplicated malaria (3·3, 1–12). All isolates from the children with cerebral malaria formed rosettes (fig 1), whereas 10 (18%) of the 57 isolates from the uncomplicated cases lacked rosetting parasites. The mean (range) in-vitro rosetting rate of isolates obtained from patients with severe malaria was significantly higher than that of isolates obtained from patients with mild forms of the disease (35%, 6–85 *vs* 17, 0–71; $p<0{\cdot}001$ Student's *t*-test), even if all non-rosetting isolates were excluded from the latter group (35% *vs* 20%; $p<0{\cdot}001$). 6 of the children with cerebral malaria (but none with mild disease) died, all within 72 h of admission. Their mean rosetting rate was high (40%, range 16–56) (fig 2) and one of the isolates formed giant rosettes—the only giant-rosetting isolate of the 81 included in this study.

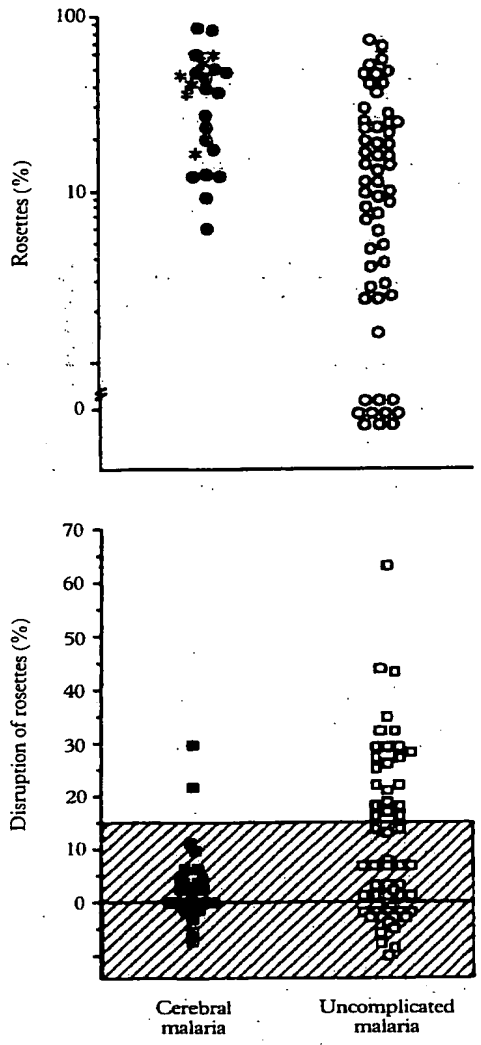

Fig 2—Frequency of rosette forming infected erythrocytes in fresh clinical isolates (top) and disruption of rosettes in vitro by human heparin depleted plasma (bottom).

* = patient died.
Values within shaded area (mean of controls ±2 SD) are not significant.

The mean parasite density was higher in the group with cerebral malaria (8·4%) than in the other group of patients (5·7%) but there was no correlation in each individual case between the magnitude of rosetting and parasite density (Spearman's rank correlation test).

Anti-rosetting activity was found in only 2 (8%) of 24 of the heparin depleted plasma samples from children with cerebral malaria (fig 2), whereas 21 (38%) of 57 of the samples from children with uncomplicated disease had the ability to disrupt rosettes in vitro. The mean disruption rate of the plasma from the children with cerebral malaria was also significantly lower than that of the children with uncomplicated malaria (4% *vs* 12%, $p < 0.05$, Student's *t*-test). Since children infected with parasite strains with high rosetting rates seemed to be at higher risk of getting severe disease than children infected with non-rosette forming parasites, we compared anti-rosetting activity in the children with uncomplicated malaria and parasites that had a rosetting rate of at least 30% with that in the children who had cerebral disease and parasites of a similarly high rosetting rate. Only 1 of 14 plasma samples from children with cerebral malaria showed anti-rosetting activity compared with 7 of 10 samples from children with uncomplicated malaria. The mean rosette disruption rate of these groups also differed significantly from each other (2% *vs* 17%, $p < 0.001$, Student's *t*-test). A similar anti-rosetting effect has also been found in a recent study in which the patients' serum reacted with his/her parasites in vitro (unpublished).

About 60% (34/56) of the rosette forming isolates were affected to some extent by mab 89, and 10% (5/56) of the rosetting isolates were completely disrupted. Likewise, immune serum BD245 partly disrupted 90% and completely disrupted 50% of the 40 isolates tested. There was no difference in the effect of the antibodies between isolates obtained from patients with cerebral disease and those from patients with mild forms of disease. However, the anti-rosetting effect of antibodies tended to be weak with isolates that expressed a high rosetting rate. Sera from 6 healthy Swedish blood donors, used as controls, gave no disruption of rosettes.

Discussion

We have shown that all our patients with cerebral malaria were infected with rosette forming *P falciparum* and that plasma from these patients generally had no anti-rosetting activity. By contrast, *P falciparum* parasites from patients with mild malaria either lacked the rosetting phenotype or had a significantly lower rosetting rate; moreover, anti-rosetting activity was frequently detected in the plasma of these patients. That this activity was always contained in the immunoglobulin fraction when sera/plasma samples were fractionated on protein A-Sepharose (ref 6 and unpublished) suggests that the anti-rosetting activity is antibody-mediated rather than an effect of drug treatment or of other serum components.

It is possible that seronegative mild cases infected with highly rosetting parasites would have progressed to severe disease if they had not received treatment. Thus, our findings suggest not only that erythrocyte rosetting is involved in the formation of cerebral malaria, but also that anti-rosetting antibodies might confer protection against cerebral disease.

That the anti-rosetting effect of monoclonal anti-PfHRP1 antibody or human immune serum tended to be weak among isolates expressing a high rosetting rate may point to high levels of expression of the rosetting ligand by some parasite strains or may indicate that another parasite-derived molecule is also involved in rosetting. Nevertheless, our results suggest that the parasite-derived rosetting ligand is relatively conserved compared with ligands associated with endothelial cytoadherence.[12]

Endothelial adherence of *P falciparum* infected red blood cells has been implicated in the induction of cerebral malaria. However, increased adhesion of infected erythrocytes to melanoma cells has not been found in isolates from patients with cerebral malaria.[13] The relative importance of different cytoadherence receptors (trombospondin,[14] CD36,[15,16] ICAM-1[17]), cytoadherence, and rosette formation in cerebral malaria has yet to be determined.

The higher rosetting rates of isolates from patients with cerebral malaria might explain the findings of Knisely et al[18] that "parasitized erythrocytes stuck together" with subsequent obstruction of capillary blood flow and of MacPherson et al[19] that "cerebral malaria patients have a higher density of packing and considerably larger proportion of tightly packed vessels than falciparum patients with non-cerebral disease". The observations of these investigators, together with our findings, suggest that erythrocyte rosetting has an important role in parasite sequestration/pathogenesis of cerebral malaria and that anti-rosetting antibodies modify the outcome of cerebral disease.

We thank Mr Idrissa Sambou for excellent technical assistance and Dr Eleanor M. Riley and Mr Chris Grummit for their kind help during the working period at the MRC Laboratories, Fajara, The Gambia. We are grateful to Dr Nick White and his collaborators, Tropical Medicine Unit, Oxford, UK, for some of the blood samples; Dr Diane Taylor, Georgetown University, USA, for the gift of mab89; Dr Peter Perlmann for encouragement and helpful suggestions on the manuscript; and Dr Åke Larsson for statistical advice.

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From The Lancet

Incompatibilities

Many are the pitfalls in the path of those commencing medical practice, and these are almost unsuspected by students who have few personal responsibilities during their hospital career. Those who prescribe under direction of seniors in the outpatient room too often are encouraged to make use of stock preparations, which save the time of the dispenser, while the teacher is apt to devote attention to interesting matters concerning physical signs, diagnosis, and prognosis rather than to an explanation of details connected with prescribing. Senior students and junior practitioners often have cause to regret this tendency of hospital training. . . . In many senses every prescription that is written may be regarded as an experiment. In terms of logic it might be considered in the form of a syllogism, where the value of the conclusion depends upon the amount of care that has been taken in framing the major and the minor premisses. So far as the patient is concerned the success of the experiment turns on the diagnostic power of the prescriber and his knowledge of the action of the remedies employed. . . . It must be admitted that appalling calligraphy and undecipherable abbreviations are often brought to our notice as examples of prescribing, and these it is impossible to defend unless it is assumed that they are written as a private code of which the particular prescriber and dispenser alone possess the key. . . . But even when the hand-writing is clear, the doses correct, and the directions plain, the ideal prescription will not have been produced unless the writer has carefully considered what may result when the different ingredients are mixed. By long experience some older practitioners may dash off a prescription that is free from offence, but dispensers and others too often have reason to smile, we hope kindly, when acids are found side by side with alkalis or with carbonates, when compounds of iron are prescribed with vegetable tinctures or infusions rich in tannin, or when insoluble compounds would inevitably be produced if the directions were followed without further inquiry. Good-natured chaff has often been directed against the blunderbuss prescription, where the number of the ingredients inevitably suggests either a vague diagnosis or a sanguine expectation that one of the crowd, no matter which, will hit the mark. . . . The tyro in dispensing or prescribing may regard these details scornfully, and may think they savour of examination catches, or of errors which no properly qualified man is likely to make. Men of greater experience will probably be discreetly silent as they ponder over the subject, ruefully recalling the errors of their youth, and perhaps realising recent slips in prescribing some of the newer synthetic compounds. Hints for improvement are valuable to many a practitioner, and when they are kindly offered we may be sure that they are kindly accepted. The saddest incompatibility in medicine is that which sometimes dissociates two groups who should be working harmoniously—the practitioners and the dispensers.

(Dec 18, 1915)

Proc. Natl. Acad. Sci. (U.S.A.), Submitted

-Medical Sciences-

Severe malaria: immunization with PfEMP1-DBL1α protects against the sequestration of *Plasmodium falciparum*-infected erythrocytes

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Word and character counts: 208 words in abstract; 47000 characters in total.

Abbreviations: ALP, alkaline phosphatase; BCIP, 5-Bromo-4-chloro-3-indolyl phosphate; DBL, Duffy binding-like; CIDR, cysteine-rich interdomain region; GST, Glutathione-S-transferase; NBT, Nitro-Blue Tetrazolium Chloride; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; SFV, Semliki forest virus; TM, transmembrane.

A family of parasite antigens known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is believed to play an important role in the binding of infected erythrocytes to host receptors in the micro-vasculature. Available data advocates the existence of a subset of very adhesive (rosetting, auto-agglutinating) and antigenic PfEMP1s implicated as virulence factors. Serum antibodies that disrupt rosettes are rarely found in children with severe malaria but are frequent in those with mild disease suggesting that they may be protective. Here we have developed a Semliki forest virus (SFV) vaccine with a recombinant gene encoding a mini-PfEMP1 (DBL1α-TM-ATS) obtained from a particularly antigenic and rosetting parasite (FCR3S1.2). The mini-PfEMP1 is presented to the host at the cell-surface mimicking the location of the native molecule at the infected erythrocyte surface. Antibodies generated by a regimen_priming with SFV RNA particles and boosting with a recombinant protein recognize the infected erythrocyte surface (immuno-fluorescence/ rosette-disruption) and prevent the sequestration of *P. falciparum*-infected erythrocytes in an *in vivo* model of severe malaria. The data prove the involvement of DBL1α in the adhesion of infected- and uninfected erythrocytes and the role of rosette-disruptive antibodies in preventing these cellular interactions. The work supports the use of DBL1α in a vaccine against severe malaria. (208 words)

Severe malaria is initiated by the sequestration of *P. falciparum*-infected (iRBC) and uninfected erythrocytes (RBC) in post-capillary venules of the brain, the lungs and other organs (1). This process may lead to excessive binding of iRBC and RBC in the micro-vasculature, blockage of the blood flow and death of the human host. Yet, only a small proportion of children develop severe disease upon infection (≈5%), suggesting the presence of a particular subset of *P. falciparum* in children with severe malaria (2). Indeed virulent parasites carry distinct qualities including strong antigenic- and adhesive features. For example, iRBCs of children with severe malaria express epitopes that are frequently recognised by serum-antibodies of children living in endemic areas (3). Further, they often bind to multiple receptors on endothelial- and erythrocyte surfaces displaying features such as rosetting and auto-agglutination, phenotypes which are less common or absent in parasites of children with mild disease (3-9).

Clinical immunity to severe disease is the first level of protection obtained against the pathogen by children living in malaria endemic areas. Data gathered suggest that antibodies specific for the adhesive antigens hinder the excessive binding of iRBC and thereby protect against severe disease. For example, sera of children with mild malaria often carry antibodies that disrupt rosettes formed by the parasite FCR3S1, while sera of children with severe malaria rarely affect rosette formation (4, 5). Further, in a large prospective study from Kenya it was found that children carrying antibodies reactive with the iRBC surface before the transmission season frequently were protected against disease during the subsequent malaria season (10). Thus, the sequestration of iRBC/RBC seems to bring about severe malaria while antibodies protect against the occurrence of the disease.

The dominant antigen and adhesin at the iRBC surface is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a polypeptide encoded by the *var* gene family present in roughly 60 copies per genome. Each species of PfEMP1 is composed of a long extracellular region, a transmembrane (TM) domain and an intracellular acidic terminal segment (ATS). The extracellular part of PfEMP1 has 2-9 domains including different Duffy-binding like domains (DBLα–ε) and cysteine-rich interdomain regions (CIDRα–γ) (11, 12). The N-terminally located part of each PfEMP1 is composed of a semi-conserved DBLα-CIDR1α head-structure. The DBL1α domain exists in all PfEMP1 species and it is the most conserved element of all the different extracellular parts of PfEMP1. CIDR1α has in previous work been found to generate immunity against a homologous challenge in the Autos monkey suggesting the usefulness of PfEMP1 in the vaccination against malaria as such (13). DBL1α is an attractive candidate for a vaccine against severe disease since it mediates adhesive features associated with severe malaria including blood group antigen A, heparan sulfate and CD35 (CR1) binding, receptors that participate in erythrocyte rosetting, auto-agglutination or endothelial binding (14-17).

The working hypothesis of this study was that the vaccination with one or a few DBL1α domains may hinder the development of severe malaria. Here we have selected a DBL1α domain from the PfEMP1 of the parasite FCR3S1.2, the expression of which has been found in cerebral malaria cases (M. Klinkert, personal communication). The iRBC surface of FCR3S1.2 has been found to be well recognized by sera of children living in endemic areas (P. Bull, personal communication) and ≈50% of Gambian children with mild malaria carry antibodies that disrupt rosettes formed by the parasite FCR3S1 (The mother clone of FCR3S1.2, ref. 4). Further, the iRBC of FCR3S1.2 displays resetting and multi-adhesive

phenotype associated with severe disease (4, 8, 9, 14, 18) arguing DBL1α of this parasite to be a candidate for the development of a vaccine.

We here show that antibodies generated by the use of recombinant Semliki forest virus (SFV)-DBL1α particles, boosted by an *E. coli* derived DBL1α fusion-protein, recognize native PfEMP1 at the live-infected RBC surface, disrupt preformed *P. falciparum* rosettes/auto-agglutinates and block the iRBC adhesion *in vivo*. We suggest that the vaccine-induced antibodies mimic those present in children protected against severe malaria.

Materials and Methods

The parasite

FCR3S1.2 was obtained by micro-manipulation cloning from FCR3S1 (18), a parasite previously cloned by limiting dilution (19). The two parasite-clones express the same PfEMP1 species at the infected iRBC surface and have the same repertoire of receptor binding but the rates are higher with parasite FCR3S1.2 which form spontaneous giant-rosettes and auto-agglutinates (13, 14, 18). The parasites were cultured according to standard methods (20). Rosetting rates of the two parasites were kept at >80%.

Animals

Balb/C mice (female 15 days old), Sprague Dawley rats (male 30 days old), and New Zeeland white rabbits (male 3 months old) were kept in the experimental animal facility of the Swedish Institute for Infectious Disease Control. All the experiments were carried out with permissions (N245/99, N246/99, N247/99, N177/01). from Stockholm north animal ethical committee.

Production of recombinant DBL1a, CIDR1a and DBL2d proteins in *E. coli*

The expression and purification of recombinant DBL1α, CIDR1α and DBL2δ and GST proteins was performed as described earlier (14). GST was cleaved away from the three recombinants by thrombin digestion at 4 °C overnight and removed by passage over a glutathione column. The quality and quantity of the recombinant proteins were determined with SDS-PAGE and spectrophotometry. The proteins were mixed with Freund's incomplete adjuvant before immunization.

Construction of mini-*var*-SFV plasmids

DBL1α, CIDR1α, DBL2δ domains and the TM (transmembrane) fragment from FCR3S1.2*var*1were PCR amplified with primers (D-41 5′-GACTAGTATGGCGACTTCAGGAGGTA-3′, and D-1 5′-GTATTTTTTTTGTTTGTCAAATTG-3′ for NTS-DBL1α; D-1.1 5′-GGATCCCGGGTATACTGAACTTATTGAGGCTA-3′ and D-1 for DBL1α without NTS; C-41 5′-GACTAGTGGTGCTAGTGGTACTAATGA-3′ and C-41.1 5′-TGTGTCGTTACTACCACTAAA-3′ for CIDR1α; L-41 5′-GACTAGTGTTAGTAAAGACACACCTAGT-3′ and 5′-TGTCTGTTCACATATATCTACA-3′ for DBL2δ; T-41 5′-CCAACAGCAGAAAGTGAGGAA-3′ and T-41.1 5′-GACTAGTTGCATATTTATCTTCGTCTGA-3′ for TM). All the PCR products were gel-purified before cloning. TM fragment was kinased and ligated downstream DBL1α, CIDR1α and DBL1δ with a Fast-link DNA ligation kit (Epicentre Technologies, Madison). To join the GST sequence upstream of DBL1α, CIDR1α and DBL2δ, the DBL1α-TM, CIDR1α-TM and DBL2δ-TM fragments were inserted

into the Spe*I* site of pET-41a-c(+) (Novagen, Madison, USA). The sequences were confirmed by sequencing. GST-DBL1α-TM, GST-CIDR1α-TM and GST-DBL2δ-TM fragments were amplified with primers SFV-4 5′-TCCCGGGAGCGCACTATTATAGCACCA-3′ and TM-41.2 5′-GCGGCCGCTGCATATTTATCTTCGTCTGA-3′ and digested with PspA*I* and Not*I*. Finally they were cloned into the SFV4.2 vector between the Not*I* and Asp*I* sites downstream of an artificial signal peptide sequence (21).

To construct the plasmid DBL1α-TM-SFV4.2, the DBL1α-TM fragment was amplified with primers DBL1 5′-GGATCCCGGGTATACTGAACTTATTGAGGCTA-3′ and T-41.1. The PCR product was digested with Sma*I* and BamH*I* and inserted into SFV4.2 pre-digested with the same enzyme set.

In vitro synthesis of recombinant SFV RNA

SFV RNAs were synthesised according to the standard protocol (22). Briefly, all the recombinant plasmids were linearised with Nru*I* followed by phenol/chloroform extraction and ethanol precipitation. The helper plasmids pSFV-helper-C and pSFV-helperS-2 were linearised by digestion with Spe *I*. 1.5 μg DNA was mixed with 50μl RNA transcription solution including rNTP mix (1 mM ATP, 1 mM CTP, 1 mM UTP and 0.5 mM GTP, from Amersham Pharmacia Biotech, Sweden), 5mM DTT, 50 U RNase inhibitor, 1mM m7(5′)ppp(5′)G (Amersham Pharmacia Biotech, Sweden), 1x Sp6 buffer (40 mM Hepes-KOH, Ph 7.4, 6 mM MgOAc, 2 mM spermidine-HCl), and 60 U Sp6 RNA polymerase. The reaction was carried out at 37 °C for 1 hour. 1 μl of the synthesised mRNA was analysed on a 0.6% agrose gel. RNAs were stored at –80 °C before use.

Expression of GST-mini-*var*-SFV at the surface of BHK21 cells

BHK21 cells were cultured until log phase and harvested by normal trypsinization followed by washing in PBS buffer devoid of Ca^{2+}, and Mg^{2+}, 10^7 cells were re-suspended in PBS and mixed with 50 μg of synthesised mRNA. The cells were electroporated according to the published protocol (22). Cells were returned into complete medium (BHK21 medium) and cultivated for an additional 1-2 days under normal conditions in a CO_2 incubator. To determine the surface expression, cells were deattached with a cell-scraper and washed once with PBS. $\approx 10^5$ cells were resuspended in 500 μl PBS and incubated with anti-GST mAb or anti-DBL1α antibodies (1:500 dilution) for 1 hour at room temperature under slight but constant rotation. After three washes with PBS, the cells were incubated with an Alexa-G488 labelled goat anti-mouse Ab (1:500 dilution; Molecular Probes, Leiden, The Netherlands) followed by three washes in PBS. The cells were finally re-suspended in 300 μl of PBS and examined under UV microscopy (Nikon Optiphot-2).

Recombinant SFV virus: packaging and purification.

Packaging of recombinant viral RNAs encoding recombinant PfEMP1 domains into rSFV particles was done using a two-helper RNA approach (21, 23). Briefly, BHK 21 cells were co-transfected with 50 μg recombinant RNA and two helper RNAs (50 μg each) transcribed from plasmids pSFV-helper-C and pSFV-helperS-2. After incubation for 24-48 h at 33 °C, medium containing recombinant virus stocks were harvested and particles were further concentrated by centrifugation through a cushion of 5 ml 20% sucrose in TNE buffer (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 0.5 mM EDTA). To determine particle titre, BHK21 cells were grown on cover-slips and

infected with particles at different dilutions. Indirect immuno-fluorescence of infected BHK 21 cells was performed and the titre was calculated based on the number of fluorescent cells and the dilution factor of the particle stock.

Animal immunization

1. Immunization with recombinant proteins. Five groups of mice (6 mice/group) were immunised (50 μg/mouse) intramuscularly with *E. coli* expressed GST-DBL1α, GST-CIDR1α, GST-DBL2δ, a mixture of the three fusion-proteins or GST alone on day 0, 14, 28, and 42. The antigens were mixed with Freund's complete adjuvant in the prime immunization and with Freund's incomplete adjuvant in the boost immunizations. Two weeks after the last boost, sera were collected from the mice and assayed for immune responses.

2. Priming with recombinant SFV particles and boosting with recombinant proteins. Mice, rats (6 animals/group/construct) and rabbits (2 animals/group/construct) were immunised sub-cutaneously three times with virus particles on day 0, 21 and 42. The injection doses were 10^7 particles/mouse, 10^8 particles/rat and $5x10^8$ particles/rabbit. The animals were boosted with recombinant proteins (50 μg/mouse, 200 μg/rat and 500 μg/rabbit) in incomplete Freund's adjuvant on day 63. Blood was collected from all animals three weeks after the last boost. Sera (both pre- and post-immunization) from each animal were analysed for anti-PfEMP1 activities.

Indirect Surface fluorescence assay

FCR3S1.2 iRBCs were collected when the majority of the parasites were at mid- to late trophozoite stage (≈26-30 h post invasion) and washed 3 times with RPMI 1640. The cells were re-suspended in 50 μl RPMI 1640 without serum. 50 μl cells were

mixed with the sera at different dilutions (1/20, 1/50, 1/100, 1/200, 1/400) and incubated at room temperature for 1 hour under careful, constant rotation. The cells were further incubated with Alexa 488 labelled goat anti-mouse, rat or rabbit Ab after three washes with RPMI 1640. Finally, the cells were washed and re-suspended in 50 μl PBS. A 15 μl cell suspension was mixed with 1 μl of ethidium bromide solution (0.5 μg/ml) and the surface fluorescence rate of iRBCs was scored under incident UV microscopy (Nikon Optiphot-2).

Immuno-blotting with recombinant proteins or purified iRBC membranes

Recombinant DBL1α, CIDR1α, DBL2δ (the GST tag was cleaved away, see above) were resolved in a 10% SDS-PAGE gel and blotted on to nylon membranes. Similarly, infected erythrocyte membranes were purified as described (18, 24) and the proteins were resolved in a 6% SDS-PAGE and blotted onto a nylon membrane. The membranes were cut into 0.5cm wide strips which were blocked with 2% BSA in 1% Tween-20-PBS overnight. Strips with recombinant proteins were incubated with sera at dilutions from 1/1000 to 1/15000 at room temperature for 1 hour while the strips holding polypeptides of infected erythrocyte membranes were incubated with serum at a 1/200 dilution. After three washes with a TBST buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween 20), the strips were incubated with an ALP-conjugated antibody (DAKO, Denmark) at a 1/1000 dilution. Finally, the antibody binding was visualised with the use of a BCIP/NTP solution (Sigma, USA).

Rosette disruption assay

Rosette disruption assays were performed as described (4, 20). Briefly, 25 μl of a FCR3S1.2 culture was mixed with an equal volume of sera at different dilutions

(undiluted, 1/2, 1/5, 1/10 and 1/20) and incubated at 37 °C for one hour. An aliquot was mixed with 1 µl of ethidium bromide (0.5 µg/ml) and the rosetting rate was examined in fluorescent microscopy as described (7, 20). In this assay, all sera were examined at the same time with the same parasite culture for each set of experiments.

In vivo adhesion blocking assay

To investigate whether anti-DBL1α antibodies may block iRBC binding *in vivo*, middle to late stage FCR3S1.2 trophozoite-infected RBCs (≈ 26-30 h) were purified from *in vitro* cultures using a magnet-based method (25). The parasites were radioactively labelled with ^{99}Tc *in vitro* (Pettersson *et al*, unpublished). ≈2x10^7 iRBCs or normal RBCs were injected intra-venously into the tail vein of the barbital anaesthesised rat immunized either with DBL1α (seven rats) or with GST (11 rats). The distribution of the iRBC was monitored using a γ-radiation sensitive screen for 30 minutes. The relative number of parasites sequestered in the lungs was calculated based on the activity of each cell and the total activity of the lungs and that of the whole animal.

Results

Construction of the immunogen: recombinant SFV holding mini-*var* genes

To determine whether *E. coli* expressed fusion-proteins corresponding to the different domains of PfEMP1 (GST-DBL1α, GST-CIDR1α, GST-DBL2δ) might generate surface reactive antibodies upon immunisation we injected five groups of mice (six animals in each group) with either of the three bacterially produced antigens, a mixture thereof or a control antigen (GST; four times with 50 µg; day 0 in Freund's

complete adjuvant, days 14, 28, and 42 in Freund's incomplete adjuvant). Although the ELISA titres of the serum specific antibodies (day 56) to recombinant DBL1α, CIDR1α or DBL2δ were high (>10^4, data not shown) they did not carry reactivity with the iRBC surface. This made us try an alternative method for the direct expression of the vaccine antigen in the host, using recombinant SFV with genes encoding different PfEMP1 domains (DBL1α, CIDR1α, DBL2δ). In order to mimic the expression of PfEMP1 at the iRBC surface we generated hybrid-genes, here called mini-*var* genes, encoding the functional domains (DBL1α, CIDR1α, DBL2δ), the transmembrane region (22 aa, TM) and a short part of the ATS-domain (108 aa; see Fig. 1A, 1B and Table 1). A signal peptide is provided for by the SFV vector. Some of the recombinant SFV mini-*var* genes also included the gene encoding glutathion-S-transferase (GST) in order to provide powerful T cell epitopes (26). To make recombinant SFV constructs we substituted the structural genes of the virus for a mini-*var* gene (Fig. 1B, Table 1). This was propagated on a plasmid separated from the genes encoding the capsid protein (helper C) and the envelope proteins (Helper S) in order to preclude virus-reformation in the host, making it safe for later human use (Fig. 2A). Recombinant virus particles were generated in BHK21 cells by co-transfection of the three mRNA templates transcribed *in vitro* from the plasmids. Virus titres of more than 10^8/ml were readily obtained (as measured by surface immune fluorescence rate after virus infection in BHK21 cells). After electroporation with *in vitro* synthesised RNA or by infection with recombinant SFV particles, the antigens were efficiently expressed and displayed on the surface of transfected BHK21 cells. More than 90% of the infected cells showed specific surface fluorescence after 20 hours (Fig. 2B). Normal BHK21 cells or BHK21 cells

transfected with irrelevant SFV constructs lacking the TM-ATS domains did not show surface fluorescence (data not shown).

Antibodies recognise PfEMP1 at the infected erythrocyte surface

In subsequent experiments a total of 144 animals (groups of six mice, rats, rabbits) were immunised with a heterologous prime-boost schedule: three times with recombinant SFV particles (days 0, 21, 42) and once with a homologous *E.coli*-produced fusion protein(s) (day 63, for more details see Table 1). Blood was collected from all the animals on day 84 three weeks after last boost.

Sera generated with the hetereologous prime-boost approach reacted both with live infected erythrocytes as well as with linear epitopes in ELISA and in immunoblotting (Fig. 3, 4). In ELISA with *E.coli* produced recombinant antigens (5 μg/ml as coating antigen, DBL1α, CIDR1α, DBL2δ purified from GST-fusion proteins) the end point-titres of all the sera were more than 1/15 000 (data not shown). The antibodies also recognised SDS-PAGE separated parasite-derived PfEMP1 obtained from FCR3S1.2 blotted onto nylon membranes (Fig. 3B). Both the anti-DBL1α sera (1/200) and the human hyper-immune plasma (BD 828; 1/400) reacted with a polypeptide of a molecular weight of ≈270 kDa previously identified as the surface-expressed PfEMP1 of FCR3S1.2$_{var1}$ but they also reacted with a slightly smaller PfEMP1 species, not present at the iRBC surface (Fig. 3B and ref. 18). The nature of the lower molecular weight PfEMP1 species is not known but it might be a truncated form of PfEMP1$_{var1}$ or a second PfEMP1 species expressed by FCR3S1.2. Neither the pre-immune sera nor the sera from the GST immunised animals did react with these molecules (Fig. 3B and not shown).

All the sera of the DBL1α, CIDR1α and DBL2δ immunised animals showed dotty fluorescence on the surface of live-infected iRBC in indirect immuno-fluorescence with FCR3S1.2 iRBCs (IFA; Fig. 4A). Sera from the immunised mice were overall more reactive than those of the rats and the rabbits. The sera of the mice immunised with the mixture of the three distinct constructs generated higher antibody titres than the sera of the animals immunised with a single construct (Fig. 4C). In contrast, rats and rabbits immunised with constructs containing DBL1α showed higher antibody responses compared to the other constructs. Control sera from animals immunised with SFV-GST (Table 1) or SFV-DBL1α-TM of parasite 3D7S8 (var_{common}, ref. 27; Table 1), similar in sequence to the *var* gene expressed by placental binding parasites, did not react or reacted only weakly with the surface of iRBC of FCR3S1.2 (Fig. 4C).

A GST sequence was in most mini-*var* genes present upstream of each domain except in one DBL1α construct. The rationale behind this was to provide known T cell epitopes to the immune system to ascertain a strong immune response. However, the results from immunizations with the three DBL1α constructs (SFV-GST-NTS-DBL1α-TM, SFV-GST-DBL1α-TM, and SFV-DBL1α) did not show any differences in the level of antibodies induced, indicating that DBL1α by itself carries sufficient helper epitopes.

Anti-DBL1α antibodies disrupt pre-formed rosettes and auto-agglutinates

The DBL1α domain of FCR3S1.2*var*1-PfEMP1 has been identified as a rosetting ligand mediating binding to a heparan sulfate-like GAG and the blood group A antigen present on human erythrocytes and endothelial cells (14, 15). Using a

heterologous prime-boost system we found that sera of mice, rats and rabbits immunised with either of three different mini-*var* gene constructs encoding DBL1α dose-dependently disrupt rosettes, giant-rosettes and auto-agglutinates of the parasite FCR3S1.2. Animals immunised with either of the CIDR1α or the DBL2δ domain showed weak serum-bound anti-rosette activities while the sera of animals immunised with mixtures of the constructs (DBL1α, CIDR1α and DBL2δ) disrupted rosettes (Fig. 5). Neither the immunization of animals with GST- or a DBL1α domain of var_{common} from the 3D7S8 clone (27) did generate antibodies that disrupted rosettes of FCR3S1.2 parasites.

Anti-DBL1α antibodies block the sequestration of iRBCs in immunised rats

In order to study the effect of anti-rosetting antibodies on the *in vivo* sequestration of iRBCs of FCR3S1.2 we challenged the immunised Sprague Dawley rats with ^{99}Tc-labelled iRBCs or normal RBCs. The whereabouts of the labelled cells in the circulation after inoculation in the tail-vein was monitored with the help of a gamma-radiation sensitive screen (Pettersson et al, unpublished). Using this *in vivo* sequestration model system it was found that the iRBCs adhered intensively in the lungs of GST immunised- and of normal control rats (Fig. 6 and data not shown). In contrast, the number of iRBC in the lungs of DBL1α immunised rats was negligible (P<0.002) and similar to that of the animals injected with normal human RBC (Fig. 6).

Discussion

An association between the rosetting phenotype of *P.falciparum* and severe malaria was proposed in 1990 by Carlson et al, a finding later confirmed and extended by several independent studies (4-9). Further, serum antibodies of children with mild malaria were found to frequently disrupt rosettes of the parasite FCR3S1 while sera of children with severe disease did not. This argues that antibodies directed to the rosetting-ligand maybe protective (4). The DBL1α domains of two species of PfEMP1 were later established to mediate rosette-formation (12, 13). Here we have prepared and tested a recombinant alpha virus-based vaccine in a heterologous prime-boost immunization regimen. It allows the induction of antibodies that prevent the sequestration of *P.falciparum*-infected erythrocytes in an *in vivo* model of severe malaria. The data demonstrate the involvement of DBL1α in the adhesion of infected- and uninfected erythrocytes and the role of rosette-disruptive antibodies in preventing these cellular interactions.

We initially failed in generating functional antibodies with *E. coli* derived proteins. To overcome this problem we turned to a primary immunisation with recombinant alpha-virus mini-*var* constructs and a subsequent protein boost. The mini-*var* constructs were designed to allow the proteins (DBL1α, CIDR1α, DBL2δ) to be expressed extra-cellularly but anchored at the cell membrane by the TM domain. In this way, the antigens are displayed at the eukaryotic cell surface as is native PfEMP1 on the iRBC surface, presumably allowing them to fold in a similar way as in the parasite *in vivo*. In a first pilot immunization experiment including 54 mice (data not shown), it was found that mice primed with recombinant SFV particles followed by boosting with a recombinant protein generated relatively higher antibody

titres than those immunized with particles alone. In subsequent experiments presented here, a protein boost was used in all groups and animals immunised showed high antibody responses. The antibodies not only recognised native PfEMP1 but were also biologically active.

Anti-DBL1α antibodies generated in mice, rats and rabbits, reacted specifically with the live-infected FCR3S1.2 RBC surface, and anti-DBL1α antibodies dose-dependently disrupted pre-formed FCR3S1.2 rosettes. The data confirm earlier findings that the DBL1α domain indeed mediates malaria rosetting and that the anti-rosette activities of human sera are mainly directed against this rosetting domain of PfEMP1. We did not see high agglutination activities with the immune sera generated (data not shown), probably due to the fact that IgG (not-IgM) antibodies predominate after the relatively long immunization period.

Antibodies generated by immunization in the presence of the N-terminal sequence of PfEMP1 (NTS-DBL1α) did not show any functional difference compared to those generated by the immunization with DBL1α alone suggesting a minor role for NTS in the antigenicity of PfEMP1. Further, antibodies generated by the immunization with a DBL1α species of $3D7_{var5.2}$ (var_{common}), a placental malaria-related *var* type (similar to $FCR3_{varCSA}$), did not carry any disruptive effects on FCR3S1.2 rosettes. This is likely due to the low sequence similarity inbetween the two DBL1α sequences. The two PfEMP1 types are not only phylogenetically distinct, but also functionally and immunogenically different suggesting that vaccines for cerebral- and placental malaria should include distinct PfEMP1 sequences.

One of the main obstacles in the development of malaria vaccines is the lack of a robust small animal model. SCID mice have been used for testing vaccination effects and sequestration studies have been possible in human-skin transplanted

animals (28). Both Aotus monkeys and Saimiri monkeys have been employed but they are not the natural hosts of *P. falciparum* and experiments can only be carried out in a few primate research centres (29) due to high costs and low availability. Here, we have used a recently developed inexpensive and robust rodent model, which has an intact immune system, and in which the sequestration of *P. falciparum* iRBC can be monitored *in vivo* without previous transplantation or other manipulations (Pettersson et al unpublished). ^{99}Tc-labelled FCR3S1.2 iRBCs are injected into the tail-vein and the sequestration is subsequently monitored in the lung vasculature through whole body scanning. This sequestration can be abolished by pre-treatment of the iRBCs with a low dose of trypsin (unpublished). Normal RBCs do not sequester and the iRBCs of the weakly adhesive parasite clone FCR3S1.6 only bind marginally in the lung-microvasculature (Pettersson et al unpublished). All the rats immunized with DBL1α constructs showed negligible sequestration of FCR3S1.2 iRBCs in the lung vasculature while animals immunized with control vectors showed extensive binding. This is the first proof that the DBL1α domain indeed mediates the sequestration of iRBC and that anti-DBL1α antibodies block the interaction between PfEMP1 and host receptors *in vivo* (heparan sulfate and blood group A antigen in this case, ref 15). A naturally developed anti-rosetting immunity after a malaria infection can thus be reproduced by vaccination.

The recombinant SFV system has only recently been tested in a malaria vaccine study (30). Unlike DNA vaccine constructs, recombinant SFV virus particles can efficiently infect many cell types, which means that more cells will be targeted. The RNA replicase encoded in each construct results in vigorous RNA replication and subsequent efficient translation yields high amounts of antigenic proteins (31). The SFV system is biologically safe, since the RNA is unable to incorporate into the DNA

genome. Further, the RNA replication promotes a cell apoptosis process that facilitates antigen presentation to the immune systems through cross-priming of dendritic cells. Because of these innate advantages of this system, high T cell and B cell responses have been seen in many studies in animal models (31-36). A HIV vaccine Phase I study in humans is underway.

The prime-boost strategy for malaria vaccination was first developed by Hill and co–workers (37). However, different prime-boost approaches give different immune responses. For example, DNA priming followed by a recombinant virus boost gives a more T cell-oriented response (38, 39) while priming with a recombinant virus and boosting with a recombinant protein usually produces better antibody responses (39). Our results confirm that the latter, priming with virus particles followed by protein boosting generates strong antibody responses.

Developing an anti-malaria vaccine with a variant antigen such as PfEMP1 is a challenge in itself since each parasite genome encodes about 60 PfEMP1 variants some of which are under sequence variation. To circumvent the difficulties, we selected a DBL1α domain of a prototypic virulent parasite (FCR3S1.2) that has previously been found to be well recognized by the sera of children living in endemic areas. For example, roughly 50% of Gambian children with mild malaria carry antibodies that disrupt the rosettes of FCR3S1 (4). Further, the iRBC surface of FCR3S1.2 is often recognised by sera of Gabonese and Kenyan children (ref. 40 and P. Bull, personal communication) and it displays the multi-adhesive phenotype associated with iRBC obtained from children with severe malaria (9, 14, 18). The DBL1α of FCR3S1.2 is by itself a vaccine candidate worth of further study but it is likely still that at least a few DBL1α domains are to be included in a vaccine that protects against severe malaria reducing the mortality of the African child.

In conclusion, we here report the first development of a prototypic anti-severe/cerebral malaria vaccine using a semi-conserved DBL1α domain of PfEMP1. The data suggests that anti-DBL1α antibodies are functional in terms of blocking iRBC adhesion *in vivo* arguing that vaccine-induced antibodies may prevent the occurrence of severe malaria.

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Figure legends:

Table 1. Dose and immunization times of different immunogens.

Figure 1.

A. Schematic outline of the primary structure of FCR3S1.2 PfEMP1 and different recombinant mini-PfEMP1s. The domains are colour-coded and the positions of the first and the last amino acid of each domain are shown under each mini-PfEMP1 construct. A GST gene was grafted upstream of NTS-DBL1α, DBL1α, CIDR1α or DBL2δ. A fragment of the *var* gene encoding 130 amino acids containing the transmembrane (TM) region and a short ATS tail were ligated down stream of each domain except in the case of the GST construct.

B. Schematic outline of the SFV vector carrying a mini-*var* gene. The position of the 26S promoter during transcription of the *var*-genes is indicated.

Figure 2. RNA synthesis and expression.

A. *In vitro* synthesis mRNA from different recombinant SFV constructs. RNA samples were analysed on a agarose gel and stained with ethidium bromide.

B. Surface expression of recombinant GST-NTS-DBL1α–TM, GST-CIDR1α–TM and GST-DBL2δ–TM on live BHK21 cells. BHK21 cells were infected with recombinant SFV particles for 18 hours and de-attached mechanically. Surface expressions of mini-PfEMP1 are shown here with an indirect fluorescence assay performed with an anti-GST mAb and Alexa G-488 labelled goat anti-mouse antibodies (see also M&M).

Figure 3. Anti-DBL1α antibodies specifically react with recombinant DBL1α or PfEMP1 extracted from *P. falciparum* infected RBC membranes (FCR3S1.2).

A. Anti-DBL1α antibodies recognise *E. coli* expressed recombinant DBL1α. Serum from a GST-NTS-DBL1α immunised rat in a 1:1500 dilution reacted specifically with DBL1α blotted onto a nylon membrane while a serum from GST immunised rat did not react with DBL1α at any dilution (lane 2, 1:1500 dilution).

B. Anti-DBL1α antibodies specifically react with *P. falciparum*-derived PfEMP1. The anti-DBL1α and the human hyper-immune IgG antibodies recognize a polypeptide of relative molecular weight of more than 250 kDa, the same PfEMP1 of FCR3S1.2 has been previously identified. A second of slightly lower molecular weight was also recognised by both anti-bodies (asterisk), but this was not expressed at the iRBC surface (data not shown). Some reactivity with the two spectrins (indicated by dots) was sometimes seen. The anti-GST antibodies did not react with any protein extracted from the parasite.

Figure 4. Anti-PfEMP1 antibodies recognise native PfEMP1 at the infected erythrocyte surface.

A. Surface fluorescence of live iRBC with antibodies generated by the heterologous prime-boost immunization regimen. FCR3S1.2 iRBCs were incubated with a rat serum from immunization with SFV-GST-NTS-DBL1α-TM particles and recombinant DBL1α. The specific antibodies that reacted with iRBC surface were detected with an Alexa G-488 labelled goat anti-rat IgG (green). The parasite was counter-stained with ethidium bromide (red). Uninfected RBCs did not give any fluorescence. The same iRBC is shown in parallel under UV (left) and phase-contrast normal light (right).

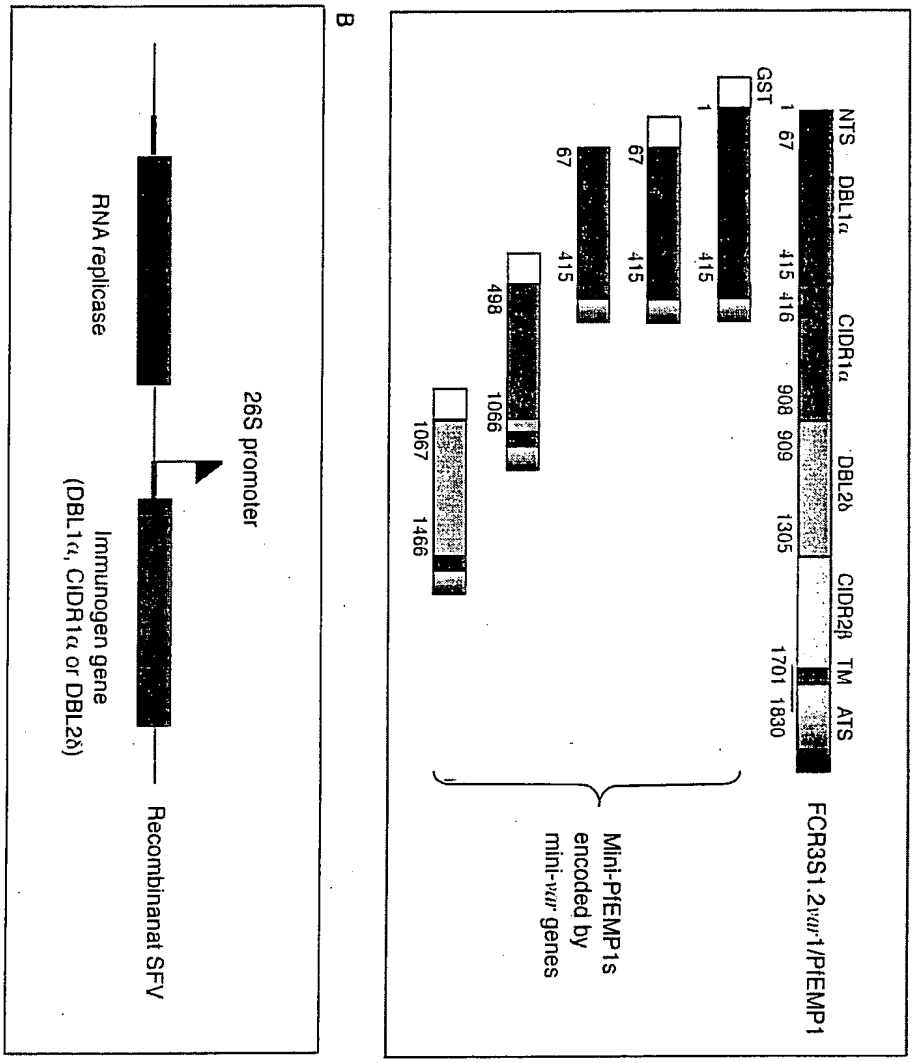
B. Surface fluorescence titres of antibodies generated by the immunization with recombinant proteins. Anti-DBL1α Abs generated with *E. coli* expressed proteins failed to recognize PfEMP1 on the FCR3S1.2 iRBC surface. Sera from fifty-five mice immunised with GST-NTS-DBL1α, GST-CIDR1α, GST-DBL2δ or GST alone did not show any surface fluorescence at 1:10 (or at other) dilutions.

C. Surface fluorescence titres of antibodies generated by heterologous prime-boost immunization of recombinant SFV particles and recombinant proteins. FCR3S1.2 infected RBCs were incubated with sera of immunized animals from dilution 1:20 to 1:400 (see M &M for further details).

Figure 5. Rosette disruptive activity of the sera generated by the heterologous prime-boost immunization regimen. An aliquote of rosetting FCR3S1.2 (rosetting rate of >80%) was mixed with sera diluted from 1:2 to 1:20 and the rosetting rate was scored in a fluorescence microscopy (see M&M for further details).

Figure 6. *In vitro* sequestration of infected erythrocytes in the lungs of the Sprague Dawley rat. The number of cells sequestered in the lungs of GST immunised rats (8 rats) was 3-4 times more than that in the lungs of rats immunised with DBL1α seven rats. The difference between the two groups was statistically significant ($P<0.002$). The number of normal human RBC in the lungs of GST immunised rats (three rats) was similar to that in the DBL1α immunized groups and the difference between the two groups was not significant ($P>0.27$) (see M&M for further details).

Figure 1



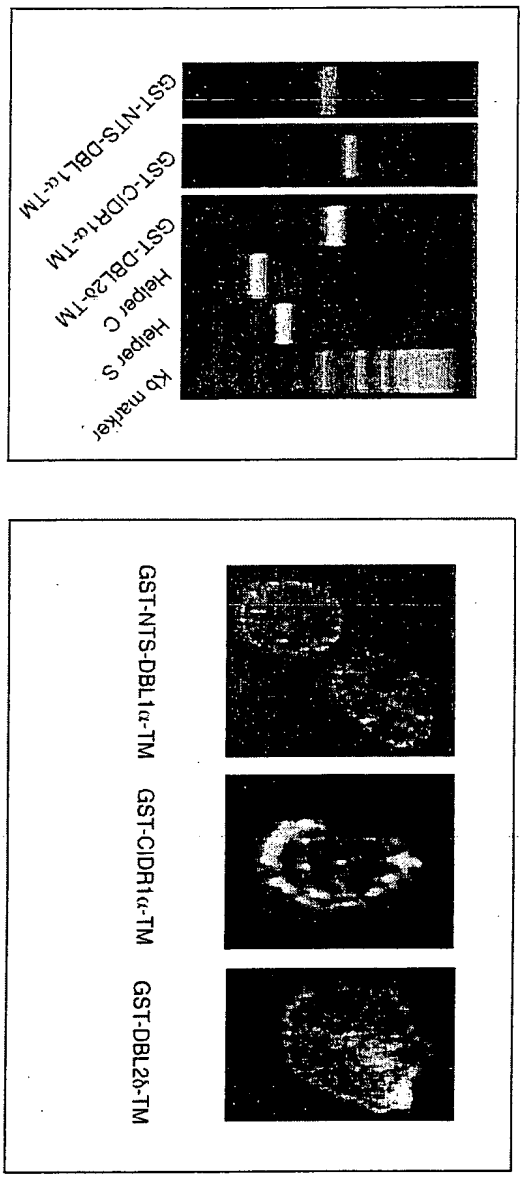


Figure 2

Figure 3

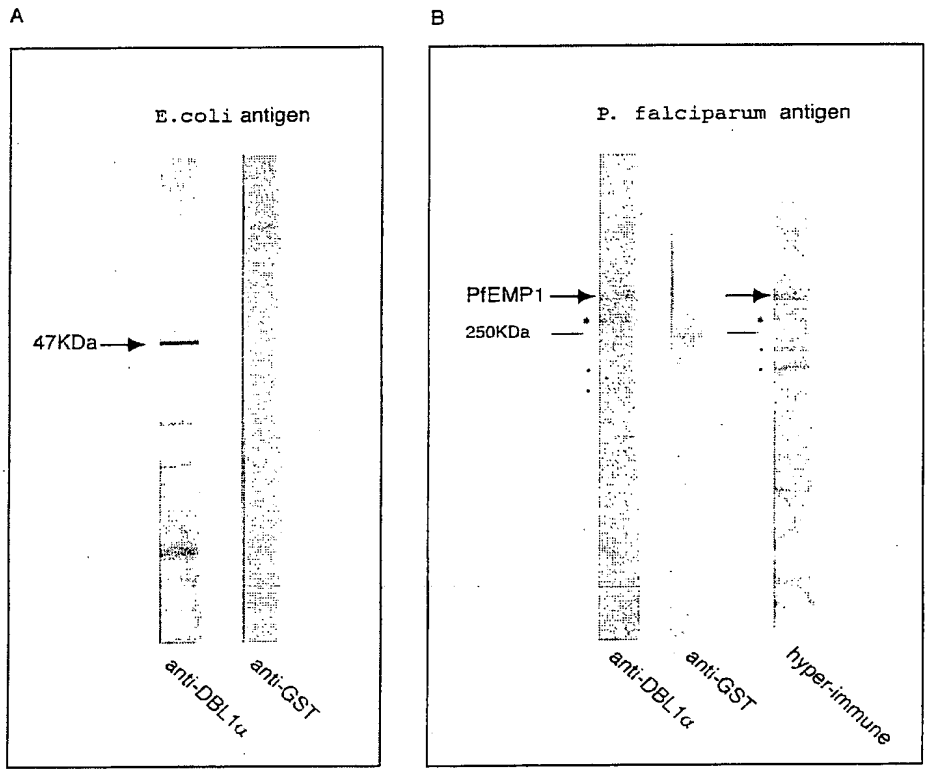


Figure 4

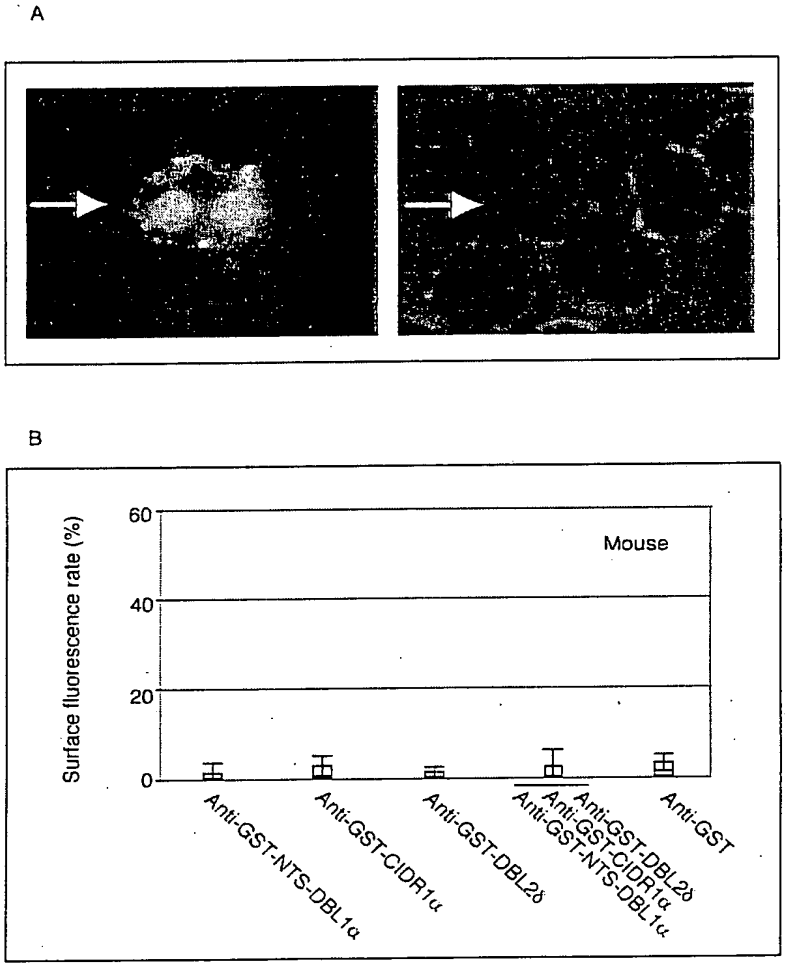


Figure 4

C

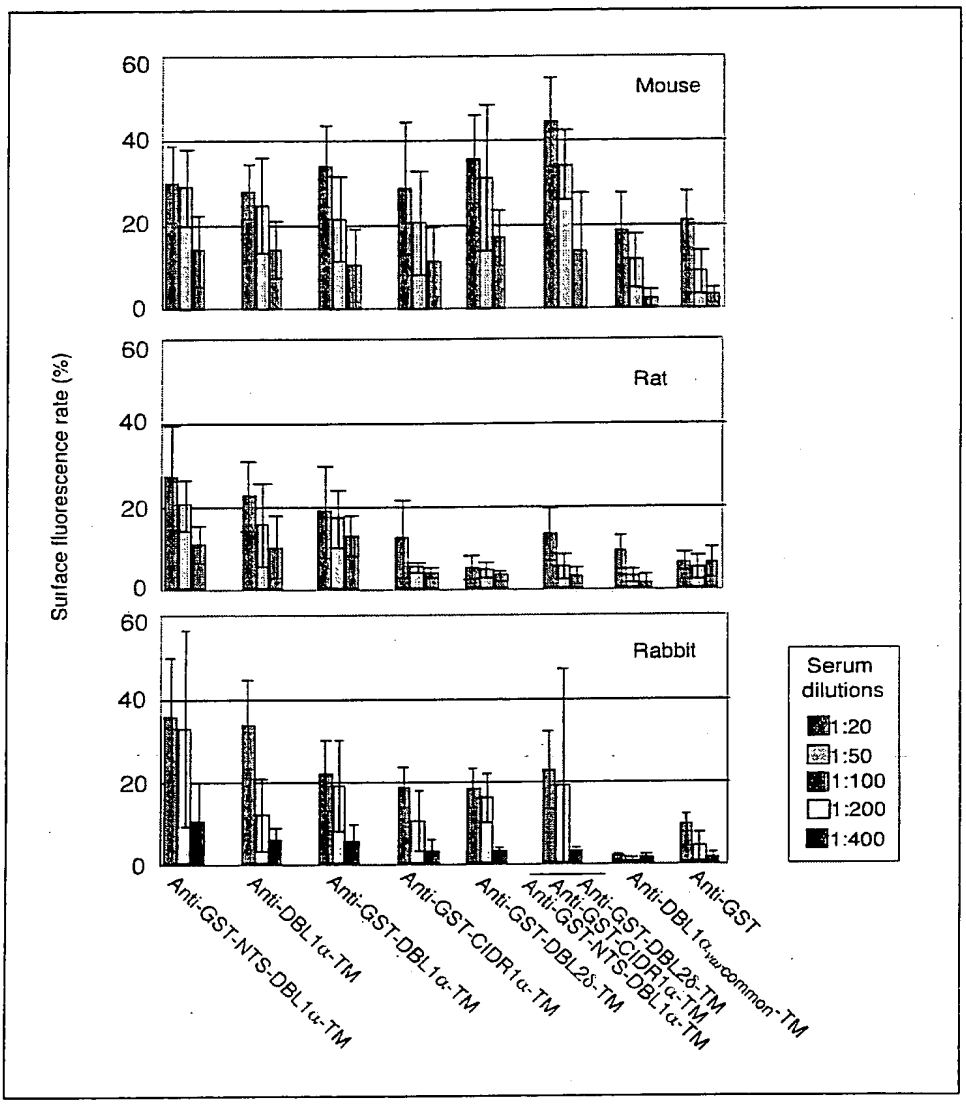
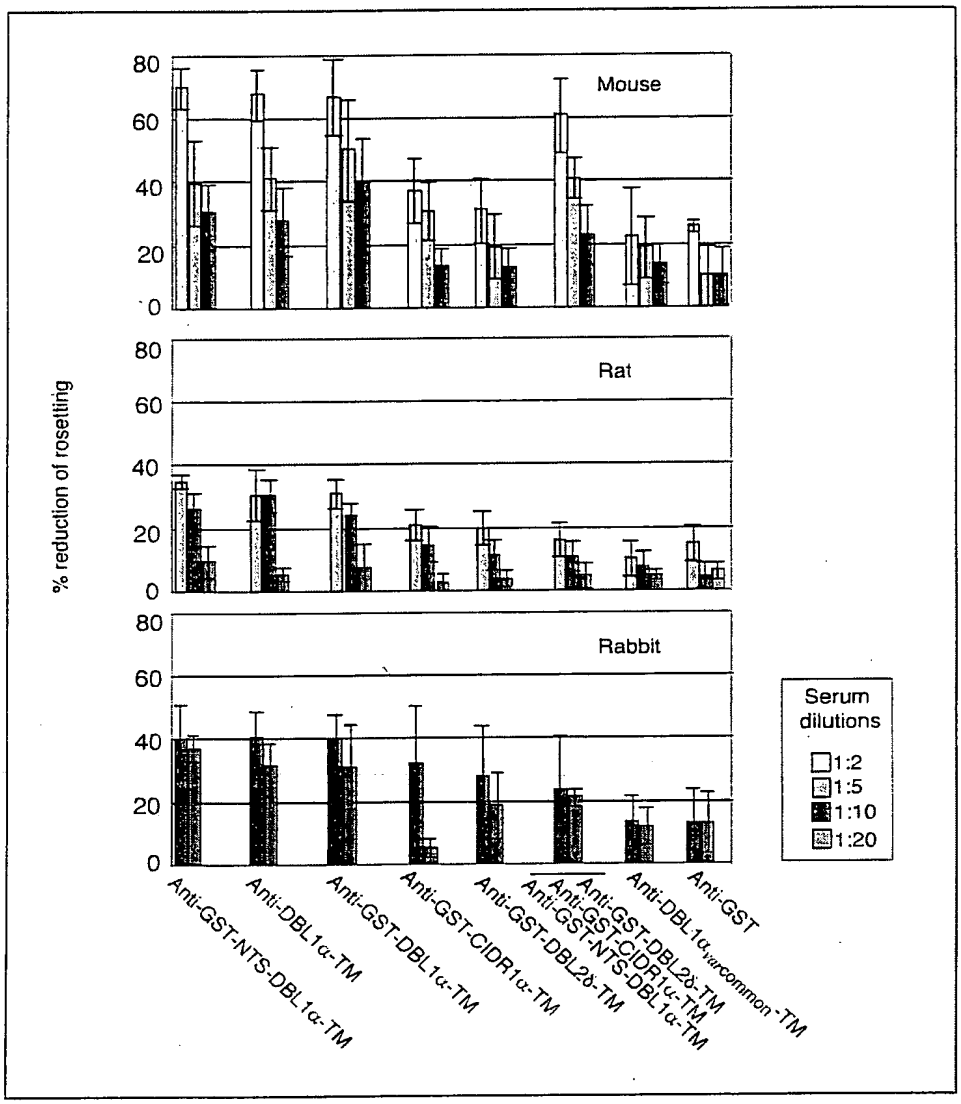


Figure 5



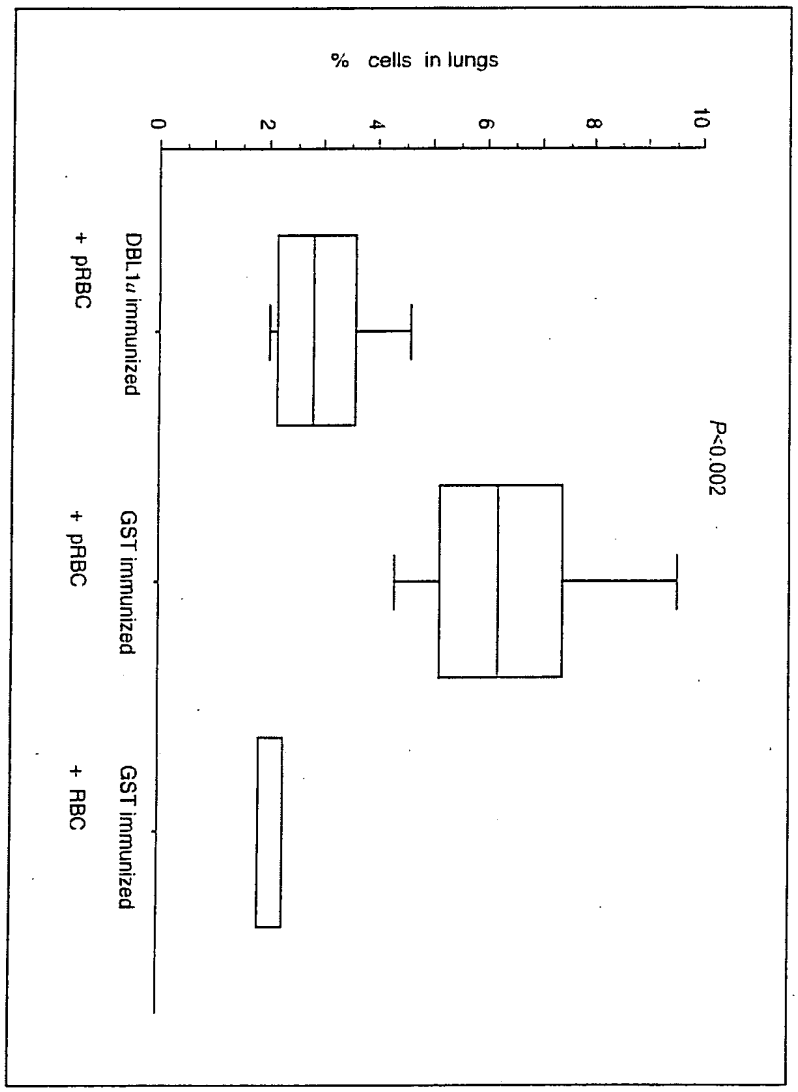


Figure 6

Table 1

	rSFV constructs/proteins	Animal Nr per group	Particle dose (particles x 3times)			Protein dose (µg x times)		
			Mouse	Rat	Rabbit	Mouse	Rat	Rabbit
Mouse/Rat/Rabbit	SFV-GST-NTS-DBL1α-TM	6	10^7	10^8	$5x10^8$	50 x 1	200 x 1	500 x 1
	SFV-DBL1α-TM	6	10^7	10^8	$5x10^8$	50 x 1	200 x 1	500 x 1
	SFV-GST-DBL1α-TM	6	10^7	10^8	$5x10^8$	50 x 1	200 x 1	500 x 1
	SFV-GST-CIDR1α-TM	6	10^7	10^8	$5x10^8$	50 x 1	200 x 1	500 x 1
	SFV-GST-DBL2δ-TM	6	10^7	10^8	$5x10^8$	50 x 1	200 x 1	500 x 1
	SFV-GST-NTS-DBL1α-TM SFV-GST-CIDR1α-TM SFV-GST-DBL2δ-TM	6	10^7	10^8	$5x10^8$	50 x 1	200 x 1	500 x 1
	SFV-GST-DBL1varcommon-TM	6	10^7	10^8	$5x10^8$	50 x 1	200 x 1	500 x 1
	SFV-GST	6	10^7	10^8	$5x10^8$	50 x 1	200 x 1	500 x 1
Mouse	GST-NTS-DBL1α-TM	6				50 x 3		
	GST-CIDR1α-TM	6				50 x 3		
	GST-DBL2δ-TM	6				50 x 3		
	GST-NTS-DBL1α-TM GST-CIDR1α-TM GST-DBL2δ-TM	6				50 x 3		
	GST	6				50 x 3		